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We exposed Xenopus tropicalis to 1, 3.3, 11 and 36 ug/L octylphenol from Neiuwkoop-Faber (NF) state 46 tadpoles through adulthood in a flow-through water system. At NF stage 5, a random subsample of froglets was collected and assessed, while the remaining frogs were exposed through 31 weeks. Significant induction of vitellogenin was observed in the high treatment at the larval subsampling for both sexes, but not at the final sampling for either sex. No significant deviation from control for sex ratio was observed for either sampling period, suggesting minimal to no effect on gonad differentiation. Oviduct development was observed in males exposed to octylphenol. These results indicate that octylphenol exposure can induce vitellogenin in immature froglets and the development of oviducts in male adult frogs. The lack of effect observed on the developing gonads suggests that in amphibians, secondary sex characteristics are more susceptible to estrogenic compounds than developing gonads.

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Effects of 4-*tert*-Octylphenol on *Xenopus tropicalis* in a Long Term Exposure

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Amphibian species are useful indicators for the effects of endocrine disruptor exposure in the environment. An experiment was conducted to determine the effects of the estrogenic endocrine disruptor, 4-*tert*-octylphenol (OP), on growth and reproductive parameters in *Xenopus tropicalis* in a flow through diluter system. Frogs were exposed to 1 μg/L, 3.3 μg/L, 11 μg/L, and 36 μg/L OP, at 34 frogs per tank, 3 tanks per concentration, and 9 tanks of unexposed frogs were used as controls. The frogs were exposed starting from stage 46 of metamorphosis through adulthood. At stage 65, approximately 6 to 7 weeks of exposure, 10 froglets per tank were randomly selected for blood collection for serum vitellogenin (VTG), sex determination by gross morphology, and gonadal histology. An induction of VTG in both males and females was seen in the 36 μg/L OP exposure compared to the control. Since VTG is an estrogen inducible yolk protein expressed at high levels in mature females, and stage 65 froglets should not have measurable VTG levels, this indicates that 36 μg/L OP was sufficient to stimulate early production of VTG. A significant increase in body length and weight for the 1 μg/L OP exposure compared to control was observed, but there was no difference in sex ratio for any of the OP concentrations. At the final necropsy, serum VTG, estradiol, testosterone, and T4 were measured, gonad, liver, kidney and thyroid tissues for histological examination were collected, and sperm counts, oocyte number, and stage were determined.

Opinions, interpretations, conclusions, and recommendations are those of the author and not necessarily endorsed by the U.S. Army.

Effects of 4-*tert*-Octylphenol on *Xenopus tropicalis* in a Long Term Exposure

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Abstract

Endocrine disrupting chemicals that activate the estrogen receptor are routinely detected in the environment and are a concern for the health of both exposed humans and indigenous wildlife. We exposed the western clawed frog (*Xenopus tropicalis*) to the weak estrogen octylphenol from Nieuwkoop-Faber (NF) stage 46 tadpoles through adulthood in order to document the effects of a weak estrogen on the life history of an amphibian species. Frogs were exposed to 1, 3.3, 11 and 36 µg/L octylphenol in a continuous flow-through water system. Just prior to completion of metamorphosis (NF 65), a random subsample of froglets was collected and assessed, while the remaining frogs received continued exposure through 31 weeks of exposure when the remaining animals were sampled. Significant induction of the female egg yolk protein precursor vitellogenin was observed in the high treatment at the larval subsampling for both males and females, but not at the final sampling for either sex. No significant deviation from the control sex ratio was observed for either sampling period, suggesting minimal to no effect of octylphenol exposure on gonad differentiation. No effects in the adult frogs were observed for mortality, body mass and size, liver somatic index, estradiol and testosterone serum levels, sperm counts, or oocyte counts. The development and growth of oviducts, a female-specific secondary sex characteristic, was observed in males exposed to octylphenol. These results indicate that octylphenol exposure can induce vitellogenin in immature froglets and the development of oviducts in male adult frogs. The lack of effect observed on the developing gonads suggests that in amphibians, secondary sex characteristics are more susceptible to impact from estrogenic compounds than the developing gonads.

Keywords: Xenopus tropicalis, octylphenol, estrogenic, vitellogenin, oviducts

1. Introduction

Endocrine disrupting chemicals (EDCs) elicit their effects by altering the normal physiological functions of various components of the endocrine system and have been shown to adversely interact with the endocrine system of a variety of organisms (Kloas, et al. 2009). EDCs have been detected in various and diverse environmental media including soil, ground water, and surface water (Barrek, et al. 2009; Duran-Alvarez, et al. 2009; Lei, Huang et al., 2009; Oehlmann, et al. 2008; Teuten, et al. 2009) and have been reported to negatively impact wildlife populations (Bernanke and Kohler, 2009; Taylor and Harrison,1999). For example, male alligators in Lake Apopka, which is contaminated with DDT breakdown products and the reproductive toxicant 1,2-dibromo-3-chloropropane（DBCP), were shown to suffer from defects in testis development (Semenza, et al. 1997). Female gastropods exposed to the androgenic EDC, tributyltin, developed an imposex condition that prevented proper reproduction in these animals (Bailey and Davies ,1988; Oehlmann, Fioroni et al. 1996). Male fish downstream from sewage treatment plants discharging estrogenic EDCs have been reported to express elevated levels of the female-specific egg yolk protein, vitellogenin (Diniz, et al. 2005).

The effects of EDC exposure have also been reported in amphibian populations, especially in anurans. Several studies have associated agricultural land use and exposure to various endocrine disrupting herbicides and pesticides with abnormalities in male gonads (McCoy, et al. 2008; McDaniel, et al. 2008; Mosconi, et al. 2005). In addition, numerous studies have demonstrated the susceptibility of the anuran endocrine system to alterations due to toxicant exposure (Hogan, et al. 2008; Storrs and Semlitsch, 2008; Mann, et al. 2009). Most of these studies focus on larval development, with few studies examining the effects of EDC exposure on juvenile development or the adult frog.

This research aims to increase our understanding of the ability of EDC's and specifically weak estrogens to interfere with normal endocrine physiology in anurans through reproductive

maturity of these animals. Additional research in amphibian environmental toxicology is important for several reasons. First, amphibian populations are in decline globally with roughly one third of all species considered endangered (International Union for the Conservation of Nature, 2009; Stuart, et al. 2004). While habitat degradation and disease are considered larger factors in this decline, investigations into the role of environmental contaminants in negatively impacting amphibian populations would increase our ability to protect these animals (International Union for the Conservation of Nature, 2009). Amphibians have a unique life cycle patterns that differ from those organisms traditionally used in environmental toxicology studies. For example, larval development of most amphibians occurs in an aquatic environment, while juveniles and adults inhabit a diverse array of environments including aquatic, terrestrial, arboreal, and subterranean habitats. The U.S. Environmental Protection Agency in developing the Endocrine Disruptor Screening Program acknowledges the importance of an amphibian model by proposing two anuran assays: one focusing on thyroid effects during metamorphosis and the other as a life cycle assay designed to detect impacts on a suite of endocrine-related endpoints (U.S.Environmental Protection Agency,1999; U.S.Environmental Protection Agency, 2007).

One of the principal mechanisms by which some EDC's elicit their effects is activation of the estrogen receptor. These chemicals are capable of mimicking endogenous estrogens and causing alterations in estrogen-responsive tissues such as the liver and gonads. Some of these chemicals are quite potent, such as 17α-ethynylestradiol, the active ingredient in some birth control formulations, which has been shown to activate estrogen receptors at lower concentrations than endogenous estrogens in some cell-based assays (Mitsui, et al. 2007). Most estrogenic EDC's, however, are weak estrogens, and activate estrogen receptors at concentrations orders of magnitude higher than that of endogenous hormones (Mitsui, et al. 2007). While these chemicals have been demonstrated to weakly activate the estrogen receptor *in vitro*, they nevertheless can elicit endocrine toxicity *in vivo* in a diverse group of organisms, including fish (Ortiz-Zarragoitia and Cajaraville, 2005; Rasmussen, et al. 2005a; Seki, et al.

2003), mammals (Laws, et al. 2000), reptiles (Semenza, et al.1997), amphibians (Kloas, et al. 1999), and invertebrates (Andrew, et al. 2008).

The weak estrogen, 4-*tert*-octylphenol (OP), (Bonefeld-Jorgensen, et al. 2007), is a degradation product of octylphenol polyethoxylate used as non-ionic surfactants in industrial and household settings (Ying, et al. 2002a). OP has been measured in surface waters of the Danube near Budapest at 1.6 – 178 ng/L (Nagy, et al. 2005), the Haihe River in China at 18 – 20 ng/L (Jin, et al. 2004), the Mai Po Marshes Nature Reserve in Hong Kong at 11 – 348 ng/L (Li, et al. 2007), and in Great Lakes sediments in the U.S. and Canada at 0.002 – 23.7 ng/g (Bennett and Metcalfe ,1998). Kloas et al. (1999) reported that exposure of larval *X. laevis* to OP at concentrations of 2.1 and 21 µg/L resulted in a significant shift in sex ratio towards females (Kloas, et al.1999).

In this study, we hypothesized that exposure of the western clawed frog, *Xenopus tropicalis*, to the weak estrogen, 4-*tert*-octylphenol, during larval and juvenile development would result in endocrine toxicities that would manifest itself at the end of larval development as well as in the adults. These toxic effects would be similar to those seen in fish exposed to estrogenic compounds including alterations in sex ratio, induced vitellogenin in males, and abnormal testicular development. The experimental setup was designed to evaluate and compare effects at two different life stages in this organism. Although endpoints measured in this study were primarily centered on reproductive parameters (e.g., gonad development); endpoints related to the overall development and health of the organisms were also measured.

2. Materials and Methods

2.1. Animals

Animals used were the western clawed frog, *Xenopus tropicalis* (Nigerian strain), originally obtained from Xenopus One (Dexter, MI) and bred for five generations in our in-house colony. Breeders were housed in partially filled 120 L tanks with a flow through rate of 5.4 turnovers per day. The aquaculture water used for housing and exposure of the frogs was prepared by mixing treated tap water with ground water at approximately a 50:50 ratio. This dilution was done to reduce the natural hardness of the ground water used for the study. The aquaculture water was then aerated, 10 µM filtered, UV sterilized and heated to 25 °C before use. Adult frogs were fed Nasco pellets (Fort Atkinson, WI) and tropical fish food flakes (Aquatic Eco-Systems Inc, Apopka, FL) once daily. A 12:12-hour light:dark cycle was maintained.

Embryos were obtained as previously described (Knechtges, et al. 2006). Briefly, ten breeding pairs were induced to mate using one injection of 200 IU human Chorionic Gonadotropin (Chorulon, Provet, Kansas City, MO) and kept overnight in the dark in 4 L breeding chambers in a 25 ± 2°C incubator. The next morning, the most viable spawn was rinsed in 2% L-cysteine at pH 8 in order to remove the gelatinous coatings in order to increase ease of handling and sorting. Non-developing or abnormally-developing embryos were removed daily. On the third day when most embryos were at Nieuwkoop and Faber (NF) stage 46 (Hubrecht-Laboratorium, et al. 1967), the embryos were randomly transferred into the exposure tanks.

Tadpoles were fed 10 mL of a 50:50 mixture of Sera Micron (Pondside Herp Supply, Indian Harbor Beach, FL) and Nasco frog brittle powder (Nasco, Fort Atkinson, WI) suspended in water (0.4 g per 10 mL water) one time on the first day of exposure, then four times per day through day 50 of exposure. Half a gram of advanced tadpole diet (ATD: 2 L finely ground fish flakes, 1 L 45% protein Nasco fish food powder and 34g Sera Micron) was added once on day 25, then four times daily and continued through metamorphosis. Half a gram of Nasco pellets were added once on day 35, then four times daily until metamorphosis. Once 95% of the animals

in a tank reached stage 66 of metamorphosis, at about week 7 of exposure for control tanks, Sera Micron, Nasco frog brittle powder, and ATD feeding was discontinued.

Once post-metamorphic frogs were observed in the tanks, 0.5 g Nasco pellets were added from day 35 to 51, 1.0 g Nasco pellets from day 52 to 61, 2.0 g from day 62 through the end of the exposure. Starting on day 51, 0.5 g fish flake food (Aquatic Eco-Systems, Inc., Apopka, FL) was given four times daily, and then increased to 1.0 g on days 52 – 61, 2.0 g from day 62 through the end of the exposure. Feeding frequency of Nasco pellets and fish flake food was decreased to three times daily on day 91, to twice daily on day 121, and finally to once daily at day 151 until the end of the exposure.

Frogs being culled and moribund frogs were euthanized by immersion for 10 minutes in 0.2% Tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO) adjusted to pH 7.0 with sodium bicarbonate (Sigma). Frogs euthanized for the interim and final necropsies were first immersed in MS-222 solution until a deep surgical level of anesthesia was reached, about 3 minutes, followed by blood collection and euthanasia by exsanguination and/or decapitation.

2.2. Environmental Conditions

The exposure was conducted in a flow through diluter system containing 15 L glass aquaria containing 9 L aquaculture water set in a water bath maintained at 25°C. Each diluter pump dispensed 300 mL aquaculture water to three replicate aquaria every five minutes with or without OP at 1.0, 3.3, 11, and 36 μg/L, to equal a flow rate of 5.4 tank turnovers per day. Diluter pump calibration was checked weekly and recalibrated as needed. All tubing used was polytetrafluoroethylene (PTFE). In order to minimize microbial colonization and degradation of OP, well water was 0.2 μm filtered at the diluter inlet, the diluter system was disinfected with 5% Minncare cold sterilant (Mar Cor, Skippack, PA) every second week, and aquaria were replaced four times during the exposure when measured OP concentrations dropped > 20% and could not be adjusted by increasing stock concentrations.

Animals were kept on a 12:12 light:dark cycle, with lighting provided by 40 watt bulbs (Duro-Test, Philadelphia, PA) providing an intensity range between 600 to 1000 lux. Water quality was maintained in accordance with ASTM standard E 1241-98 (American Society for Testing and Materials International, 2004), and measured weekly. Water quality readings during the study ranged from 23.4 to 25.7°C for temperature, 0.7 to 7.1 mg/L for dissolved oxygen, 571 to 760 Siemens/cm for conductivity, 7.25 to 7.77 for pH, 100 to 143 mg/L (as $CaCO_3$) for alkalinity, 0.22 to 3.18 mg/L for ammonia, and 176 to 218 mg/L (as $CaCO_3$) for hardness (Table 1).

2.3. Test Compound

Since 4-*tert*-octylphenol (Sigma) has low solubility in water (Ying, et al. 2002a), a saturation column system was used to generate a super-stock solution used for making the daily working stock solutions. The saturation column system was set up as previously described (Kahl, et al. 1999). Glass wool columns were constructed from 1.2 m lengths of medium wall (25 mm o.d., 23 mm i.d.) glass tubing (Kimble Kontes, Vineland, NJ) bent over flame into a "U" shape and stuffed with glass wool (Daigger, Vernon Hills, IL). OP was dissolved in acetone (5 g in 60 mL), applied to both sides of the column and dried down under vacuum. The column was flushed by running Milli-Q water (Millipore, Billerica, MA) through at 20 mL/min for 18 – 24 hours, then the flow rate was reduced to 4 mL/min for 2 - 4 days, during which samples of the eluate were taken for determination of OP concentration. When put into use, the column was moved into a 25°C incubator and connected to a low-flow pump (FMI, Syosset, NY) running at 4 mL/min. Eluate was collected in a glass Erlenmeyer flask and used to make daily working stocks A (1100 μg/L) and B (270 μg/L) based on the measured concentration of octylphenol (typically around 11 mg/L). Columns were replaced when the measured OP concentration dropped below 8.5 mg/L, or about every 6 – 8 weeks. Stocks A and B were further diluted with aquaculture water in the diluter system to yield nominal tank concentrations of 11 and 36 μg/L, and 1.0 and 3.3 μg/L, respectively. Stock concentrations were adjusted based on measured tank concentrations to compensate for degradation of OP in the tank water.

Samples of super-stock and of stocks A and B were taken daily, tank samples were taken weekly, and all samples were submitted weekly for determination of OP concentration by high pressure liquid chromatography (HPLC). On the day of collection, stock samples were stabilized by filtration through 0.45 µm PTFE syringe filters (Millipore) and dilution 1:4 with acetonitrile (Optima grade, Fisher Scientific, Pittsburg, PA), and stored at room temperature until analysis. Tank samples were filtered through 100 mm diameter glass fiber filters (Fisher Scientific) under vacuum to remove particulates and submitted for analysis the same day. Samples expected to contain >15 µg/L were injected directly, and samples expected to contain <15 µg/L were concentrated by the extracting 500 mL of sample using Bakerbond C18 SPE columns (J.T. Baker, Phillipsburg, NJ). The retained OP was eluted with 2.5 mL of acetonitrile, and diluted with 7.5 mL water to yield sample concentrations assumed to be >15 µg/L. Each sample was run in triplicate and 100 µL was injected onto a Hypersil ODS C18 column (Agilent, Palo Alto, CA) in an Agilent 1100 series HPLC with a diode array detector at 195 nm, using a 50% acetonitrile: 50% water acidified with 0.2% phosphoric acid mobile phase in an isocratic elution. The flow rate was 1 mL/min and the retention time under these conditions was approximately 5 min. OP concentrations were determined using linear regression ($R^2 \geq 0.99$) from a seven point standard curve from standards ranging from 32.5 to 2000 µg/L and prepared in 25% acetonitrile: 75% water. The lowest detection limit for OP was 14.1 ± 2.6 µg/L, within run precision was 4.2%, between run precision was 9.0%, and added-mass had 100.4% recovery.

2.4. Experimental Design

The animals were exposed to 1.0, 3.3, 11, and 36 µg/L OP from NF stage 46 until 31 weeks of exposure (25 weeks post metamorphosis) was reached. There were nine replicate unexposed control tanks and three replicate tanks per OP concentration. Each tank initially contained 34 tadpoles. Ten froglets per tank were randomly sub-sampled at stage 65, during weeks 5 – 7 of exposure, and the remaining animals were sampled at 31 weeks of exposure. OP concentrations were chosen for reproductive effects based on studies using *Xenopus laevis*

(Huang, et al. 2005), *Rana catesbeiana* (Mayer, et al. 2003), *Rana pipiens* (Crump, et al. 2002; Mayer, et al. 2003), medaka (Seki, et al. 2003), zebrafish (Ortiz-Zarragoitia and Cajaraville ,2005), and a short term range find exposure for general toxicity using *Xenopus tropicalis* done in-house (data not shown).

2.5. Interim and Final Necropsy

Sampling for the interim necropsy took place during weeks 5 through 7. On the first day that an NF stage 65 froglet was seen (day 38 of exposure) the number of NF stage 65 froglets was counted in each tank. A random number between 1 and 6 was generated by throwing a die, then that number of stage 65 froglets was randomly taken from each tank for euthanasia and necropsy. This was repeated each day until 10 froglets per tank had been taken, a period of 14 days. The froglets were immersed in a $NaCO_3$ buffered 0.2% MS-222 solution (pH 7) until a deep surgical level of anesthesia was reached. The froglets were weighed and body length from snout to vent was measured. Blood was collected by cardiac nicking and sex phenotype was determined by examination of gonads under a dissection microscope; ovaries were distinguished by the presence of lobes interspersed with melanin, and testes were distinguished by their shorter length and lack of pigment (Hayes, et al. 2006). The area encompassing the gonad and kidney was fixed in Modified Davidson's Fixative (14% ethanol, 37.5% formalin, 6.25% glacial acetic acid, 42.25%. water; (Electron Microscopy Sciences, Hatfield, PA). After 4 days, fixed tissue was rinsed in water, and then stored in 10% normal buffered formalin (NBF; Sigma). The blood was allowed to clot for at least 30 minutes and centrifuged for 10 minutes at 10,000 g at 4°C, then the serum separated and stored at -80°C until analysis.

For the final necropsy at 31 weeks of exposure the order of tanks was randomized and one frog at a time was randomly chosen for euthanasia and necropsy. The order of tanks was re-randomized for each sequence of tanks until no animals remained, over a period of 6 days. The frogs were immersed in a $NaCO_3$ buffered 0.2% MS-222 solution, pH 7, until a deep surgical level of anesthesia was reached. The frogs were weighed and body length from snout to vent was

measured. Blood was collected by foot amputation; sex phenotype was determined by gross examination of gonads. The gonads, oviducts, livers, and kidneys were removed and weighed. After weighing, gonads from one third of the animals were randomly selected for sperm or oocyte counts, and the gonads not selected fixed in Davidson's Fixative (30% ethanol, 20% formalin, 10% glacial acetic acid, 10% glycerol, 30% water). Livers and kidneys were fixed in 10% NBF. The head was removed and fixed in Davidson's fixative for later dissection of thyroid tissue. After 4 to 8 days, Davidson's fixed tissue was rinsed in water, and then stored in 10% NBF. The blood was allowed to clot for at least 30 minutes and centrifuged for 10 minutes at 10,000 g at 4°C, then the serum separated and stored at -80°C until analysis.

2.6. Sperm and Oocyte Counts

As previously described by Olmstead, et al. (Olmstead, et al. 2009), testes selected for counting were placed in 15 mL centrifuge tubes containing 3 mL modified Ringer's solution (100 mM NaCl, 5 mM HEPES, 2 mM KCl, 0.1 mM EDTA, 1 mM $MgSO_4$, 2 mM $CaCl_2$, pH 7.8) and stored for up to 7 days at 4°C until analysis. On the day of analysis, the testes were homogenized using a Fisher Powergen tissue homogenizer for 2 x 30 seconds on ice. The homogenate was diluted 1:10 with modified Ringer's solution and 10 µL was pipetted onto each side of a hemocytometer. Four 1 mm squares were counted, averaged and multiplied by 0.15 to obtain million sperm per testis which was then normalized to testis weight in mg to yield million sperm per mg testis. Each sperm count was repeated if the coefficient of variation (CV) between the squares was >10% and the count with the lower CV was reported.

Ovaries selected for counting were placed in a flask containing 20 mL oocyte Ringers 2 (OR-2) buffer (1 part solution A (825 mM NaCl, 25 mM KCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM HEPES, 38 mM NaOH, pH 7.8), 1 part solution B (10 mM Na_2HPO_4), 8 parts water) (Sive, et al. 2000) with 2 mg/mL collagenase (Sigma), and incubated on a shaker for 1 hour at room temperature until no oocyte clumps remained. An additional 50 mL OR-2 buffer was added and oocytes were filtered through a 100 µm cell strainer (BD Falcon, San Jose, CA) and rinsed with

another 50 mL OR-2. The oocytes were poured into a 60 mL plastic sample cup, fixed in modified Davidson's fixative and stored at room temperature until ready for analysis. At time of analysis, the cells were poured into a 19 cm crystallizing dish with a numbered grid attached to the bottom, and enough OR-2 to cover the bottom. The oocytes were dispersed evenly, 10 random squares were selected, and the oocytes contained within were pipetted and placed in a 100 mm Petri dish. The oocytes were counted under a dissection microscope and staged as I/II, III, IV, and V/VI based on the staging criteria of Dumont (Dumont,1972).

2.7. ELISA and Radioimmunoassay

Vitellogenin concentration in serum was determined by competitive ELISA as previously described (Olmstead, et al. 2009). Briefly, medium binding EIA plates were coated with purified *X. tropicalis* vitellogenin overnight, and then normal goat serum was used to block nonspecific sites. Primary anti-vitellogenin serum preabsorbed with normal male *X. tropicalis* serum was diluted 1:4,000 in assay buffer and serum samples were diluted in assay buffer, typically 1:300 for males and 1:1000 for females, which express higher levels of vitellogenin. A standard curve was prepared with purified vitellogenin diluted with assay buffer to 12,800, 6,400, 3,200, 1,600, 800, 400, and 200 ng/mL. Samples and standards were incubated with primary anti-serum and added to the plate in duplicate. Goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) was used as a secondary antibody at a 1:20,000 dilution. Plates were incubated with tetramethylbenzidine substrate (Sigma Aldrich) and the reaction was stopped after 10 min with 1 M phosphoric acid. Absorbance was measured at 450 nm in a plate reader.

Serum estradiol and testosterone concentrations in frog serum were measured by radioimmunoassay (RIA) using methods previously described (Olmstead, et al. 2009). Steroids were extracted twice from serum with diethyl ether, evaporated to dryness, and reconstituted in assay buffer (0.1M phosphate buffered saline, 1% bovine serum albumin, pH 7.6). Sample aliquots were mixed with estradiol (Endocrine Sciences, Calabasas Hills, CA) or testosterone (Fitzgerald Industries International, Concord, MA) antibody and tritiated tracer (Amersham Life

Sciences, Arlington Heights, IL). After incubation, unbound steroids were removed with dextran-coated charcoal and the remaining bound tracer was measured with a scintillation counter. Sample responses were compared to standard curves prepared with known steroid concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.020, and 0.010ng/mL. Detection limits were calculated for each run as the lowest standards that consistently differed from the zero standards, i.e. the 95% confidence levels did not overlap.

2.8. Histology

Tissues for histological examination were fixed in Davidson's fixative and stored in 10% NBF until processing for paraffin embedding, sectioning, and hematoxylin and eosin staining. Testicular tissues were examined for the presence of seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, unusually large or small seminiferous tubule lumen, presence and/or number of testicular oocytes, sloughed germ cells located within the tubular lumen and collecting ducts, cellular infiltration into the interstitial space, Leydig cell hyperplasia or hypertrophy, increased interstitial fibrous connective tissue and events typically observed during spermiogenesis (nuclear condensation and elongation) and spermiation. Liver tissues were examined for hepatocyte vacuolization, myeloid cell proliferation, granulomatous and lymphocytic cellular infiltrate, and congestion. Kidney tissue was examined for mineralization, granulomatous and lymphocytic cellular infiltrate, proteinaceous fluid and renal tubule dilation.

2.9. Statistics

Data were calculated from the means of nine replicate tanks for controls and three replicate tanks for exposed groups and analyzed using ANOVA with Dunnett's t-test and Tukey's HSD when multiple comparisons were required. P values ≤ 0.05 were considered significant. Data for proportion of females were arcsine transformed prior to analysis. Statistics were run using SYSTAT v. 12 (SYSTAT Inc., Chicago IL) and SPSS v. 16 (SPSS Inc., Chicago IL). All data are presented as plus or minus the standard deviation except where noted.

3. Results

3.1. Analytical Chemistry

Samples for chemical analysis of OP concentration by HPLC were collected daily from stocks and weekly from frog tanks in the diluter. Averaged across the entire study, tank OP levels were 1.2 ± 0.5, 3.5 ± 0.7, 10 ± 2, and 36 ± 7 µg/L for the nominal targets of 1.0, 3.3, 11, and 36µg/L, respectively (Suppl. Table.1). Microbial degradation of OP did result in tank concentrations significantly below nominal during times of increased organic load in the tanks, particularly during weeks 5 – 7 when the mixture of tadpoles and froglets in the tanks required excess feeding (Suppl. Table 1). To the maximum extent possible the stock concentrations were adjusted to compensate for degradation, and the tanks were changed out periodically.

3.2. General Toxicity Measures

Percent spontaneous mortality was 10.4%, 5.3%,18.6%, 1.0%, and 10.6% for unexposed controls, 1.0, 3.3, 11, and 36 µg/L OP exposed animals, respectively. Total spontaneous mortality was 9.5% including all groups through the end of the study and there were no spontaneous deaths after 60 days of exposure. A Kaplan-Meier log rank analysis was performed, treating accidental deaths and euthanasia deaths for interim and final necropsies as censored data (Fig. 1). Animals exposed to 11 µg/L OP had significantly decreased mortality compared to controls, ($p \leq 0.001$). The other exposure groups were similar to the control group, indicating that there was no significant mortality associated with OP exposure.

Frogs in the interim and final necropsies were weighed and measured for body length from snout to vent. Interim necropsy lengths and weights for both male and female frogs in OP exposed groups were similar to weights in unexposed controls (Table 2), and there was no difference between exposed and control male frogs nor between exposed and control female frogs within all groups. Additionally, final necropsy lengths and weights for both male and female frogs in OP exposed groups were similar to weights in unexposed controls (Table 2), and frogs in all groups displayed species characteristic sexual dimorphism in body size. These data indicate

that OP exposure had no effect on body growth, sexual dimorphism, or size at completion of metamorphosis or maturity.

3.3. Sex Ratios

Sex phenotype of frogs sampled in the interim necropsy was determined by examination of the gonadal tissue under a dissection microscope. Percent female was 55.6 ± 20, 63.3 ± 5.8, 53.3 ± 15, 60.0 ± 26, and 56.7 ± 23 for controls, 1.0, 3.3, 11, and 36 µg/L OP exposed animals, respectively. The percent female of the octylphenol exposed frogs was similar to the unexposed control animals indicating that octylphenol exposure had no effect on sex phenotype at stage 65 of metamorphosis. Additionally, the proportion of females seen in the octylphenol-exposed frogs was similar to the unexposed control animals.

Sex phenotype of frogs sampled in the final necropsy was determined by gross examination of the gonadal tissue. Control percent female and proportion of female data were similar to that observed in the larval sub-sampling. The percentage of females in the frogs exposed to 11 µg/L octylphenol was significantly higher than the unexposed controls ($p < 0.05$, Figure 2). While this would suggest feminization of the male gonads, this effect was not observed in the highest treatment. Additionally, sex determination of the gonads in these animals is fixed during the larval stage (Miyata, et al. 1999), and gonads would not be expected to be susceptible to sex reversal following completion of metamorphosis. Since this effect was also not observed in the larval sub-sampling, this suggests that the significant effect observed in the 11 µg/L group may be a statistical anomaly and may not be biologically significant.

3.4. Testes

Testes in the final necropsy were removed, weighed, and randomly selected for either fixation for histological examination or homogenization for sperm counts. Weights of both testes in mg were 24.6 ± 2, 26.7 ± 2, 26.6 ± 1, 24.2 ± 3, and 28.1 ± 1 for controls, 1.0, 3.3, 11, and 36 µg/L OP exposed animals, respectively. Testis weights in the octylphenol-exposed frogs were

similar to the testis weights in the unexposed control frogs, indicating that OP exposure had no effect on testis weight. The sperm counts in frogs exposed 11 µg/L octylphenol were significantly lower than the unexposed controls ($p < 0.05$, Fig 3). This effect, however, was not observed in the highest treatment group.

Testes were examined for the presence of seminiferous tubule atrophy, germ cell degeneration, abnormal germ cell development, multinucleate cell formation, seminiferous tubules with unusually large or small lumen, spermatocysts content, presence of testicular oocytes, non-recognizable gonadal tissue, cellular infiltration into the interstitial space, Leydig cell hyperplasia , hypertrophy, increased fibrous connective tissue located in the interstitial space or events typically observed during spermiogenesis and spermiation. The seminiferous tubules of control animals were lined with spermatocysts containing spermatogonia, spermatocytes and spermatids at various stages of spermatogenesis/spermiogenesis. Released elongated spermatids were observed in the seminiferous tubule lumen in control and OP exposed groups. Expected octylphenol exposure related effects were not seen. The most predominant effects observed were sloughing of the seminiferous epithelium (Figures 4A and 4B) into the seminiferous tubular lumen and the increased appearance of unidentified spherical-like bodies associated with released elongated spermatids (Figures 4C and 4D).

Focal very mild to mild sloughing of the seminiferous epithelium was observed in the control, 1, 3.3, 11 and 36 µg/L OP exposed groups and severe multifocal sloughing was observed in all OP exposed groups (Table 3). There was significantly increased multifocal germ cell sloughing observed in the 1 µg/L and 36 µg/L OP exposed groups, ($p \leq 0.05$, Table 3). Small dark spheres which resemble residual bodies were observed associated with the elongated released spermatids in both the control and exposed frogs (Figures 4C and 4D). The spheres appeared to be observed more frequently in animals at the higher concentration levels and it appears that these spheres may be early rounded spermatids which have been sloughed or prematurely released into the seminiferous tubule lumen (Fig 4E).

3.5. Ovaries

Ovaries in the final necropsy were removed, weighed, and were randomly selected for either fixation for histological examination or collagenase digestion for oocyte counts and staging. Ovary weights, oocyte number, and percent oocytes at stages I - II, III, IV, and V - VI for octylphenol exposed frogs were similar to unexposed controls (Table 4). There was a slight, nonsignificant trend toward decreased ovary weights with increasing OP concentrations, but these data indicate that octylphenol at the concentrations used had no significant effect on ovary endpoints.

3.6. Non-Gonadal Histology

Liver and kidney tissue in the final necropsy was fixed in 10% normal buffered formalin, mounted in paraffin, and slides stained with hematoxylin and eosin. In the liver, moderately to severely vacuolated hepatocytes expanded by intracytoplasmic clear space suggestive of lipid accumulation were seen in unexposed controls and all exposed groups. In the kidney, the proximal tubules, collecting ducts and interstitial spaces had varying amounts of mineralization seen in unexposed controls and all exposed groups. Liver and kidney somatic indices for both male and female frogs in octylphenol exposed groups were similar to those measured in unexposed controls (Table 2). These findings indicate that there were no exposure related effects in the liver and kidney.

3.7. Vitellogenin

An ELISA assay for vitellogenin levels was performed on serum collected from the interim and final necropsies. In the interim necropsy, vitellogenin levels in frogs exposed to 36 µg/L octylphenol were significantly increased compared to unexposed controls (Fig 5). There was no difference in vitellogenin levels between male and female frogs within all groups. At 31 weeks of exposure the frogs were sexually mature, so the characteristic sexual dimorphism was seen in vitellogenin levels in all groups. Female vitellogenin levels in mg/mL were 2.3 ± 0.3, 2.4 ± 0.2, 2.4

± 0.03, 2.5 ± 0.3, and 2.3 ± 0.3 for controls, 1.0, 3.3, 11, and 36 µg/L OP exposed animals, respectively, and were not significantly different between controls and OP exposed animals. Vitellogenin levels in octylphenol exposed phenotypic male frogs were similar to levels seen in unexposed control frogs, but there was an upward trend seen in the OP exposed frogs similar to that seen at the interim necropsy that was not significant due to high variability (Fig 5). These data indicate that octylphenol can induce increased vitellogenin levels in immature froglets, but may not in sexually mature frogs.

3.8. Sex Steroids

RIA assays for testosterone and estradiol were performed on serum collected from the final necropsy. Testosterone levels in octylphenol exposed frogs were similar to levels seen in unexposed control frogs for both female and male phenotypes (Table 5). Estradiol levels in octylphenol exposed frogs were similar to levels seen in unexposed control frogs for both female and male phenotypes (Table 5). Since at 31 weeks of exposure the frogs were sexually mature, the characteristic sexual dimorphism was seen in testosterone and estradiol levels in all groups. These data indicate that octylphenol exposure has no effect on peripheral testosterone and estradiol levels in sexually mature frogs.

3.9. Male Oviducts

An incidental observation made during the course of the study was the presence of oviduct tissue in octylphenol exposed males (Fig 6). Oviducts are female specific secondary sex characteristics that function in the final maturation of oocytes just prior to mating (Wake and Dickie ,1998). This tissue is derived from Mullerian ducts that are present in both larval males and females. In males these ducts regress while in females they differentiate into the oviducts. The growth of these tissues in females is estrogen-dependent, and there was a significant increase in oviduct weight in 36 µg/L OP exposed animals compared to 11 µg/L OP exposed animals ($p \leq 0.05$), indicating a possible concentration dependent relationship between OP exposure levels and oviduct weight (Fig. 7).

4. Discussion

A particular challenge in this study was the degradation of OP in the test tanks. OP is readily adsorbed to sediments and sewage sludge, and degrades in the water column under primarily aerobic conditions (Ying, et al. 2002b). Breakdown products of OP exposed to inoculants from industrial or municipal wastewater treatment plants have been shown to degrade by 72% within 28 days with a half-life of 12 to 18 days (Staples, et al. 1999). During the study, tanks contained variable amounts of uneaten food, feces, algae from Sera Micron, and microbial contamination, all of which can contribute to adsorption and biodegradation of OP. Measures were taken prior to initiation of the study in order to minimize microbial contamination and biofilm formation, and thus OP biodegradation, including UV disinfection and triple filtration of aquaculture water, using 0.22 μm filtered Milli-Q water to make stocks, disinfection of the diluter system tubing and pumps with Minncare cold sterilant prior to the study and biweekly, and replacement of the tanks during the study. These measures reduced OP loss in the tanks by approximately 45% compared with the losses seen in a preliminary study where these measures were not taken. By using flow-through conditions where fresh OP containing water was continually introduced and taking additional measures to reduce microbial degradation in the test system, we hoped to reduce the loss of OP, but nonetheless the OP concentration appears to have been reduced during at several phases at the study.

Generalized OP toxicity was not seen in any of the exposed groups. Overall mortality was less than 10%, and there was no significant increase in mortality in any of the exposed groups compared to controls. Measures of body length and weight were not significantly different from controls, in both the interim and final necropsies. Liver and kidney weights and somatic indices in exposed animals were not significantly different from controls, and there were no histological differences seen between exposed animals and controls. These data indicates that the other effects seen in OP exposed animals were not due to general toxicity, and that the highest OP

concentration used, 36 µg/L, was at or below the no observed effect level (NOEL) for general toxicity in *X. tropicalis*.

Exposure of *Xenopus* tadpoles to endogenous estradiol was first shown to alter gonad differentiation by feminization of male gonads in the 1950s (Chang and Witschi ,1956; Gallien,1953). Exposure of *X. tropicalis* to the strongly estrogenic compound, ethynylestradiol, has been reported to feminize sex ratios in tadpoles and result in a permanent shift in the adults (Gyllenhammar, et al. 2009). Alterations in sex ratios towards females have been observed in anurans following larval exposure to weakly estrogenic chemicals (Cong, et al. 2006; Fort, et al. 2004; Kloas, Lutz, et al.1999; Levy, et al. 2004; Mackenzie, et al. 2003; Mosconi, et al. 2002; Qin, et al. 2003). Kloas et al. (1999) reported that exposure of larval *X. laevis* to octylphenol at concentrations of 2.1 and 21 µg/L resulted in a significant shift in sex ratio towards females. In the present study, no significant increases in percent female were seen between OP exposed groups and controls in the stage 65 larval sampling. However, in the adult sampling, a significant increase in percent female was seen in animals exposed to11 µg/L OP, but not in the high concentration of 36 µg/L OP compared to controls. There appeared to be a slight, non-significant trend toward a higher incidence of the female phenotype which would be consistent with reports in the literature and the known mechanism of action of octylphenol. The lack of a significant effect observed in this study may be due to a lack of statistical power compared to other studies

Vitellogenin is an estrogen-inducible yolk protein normally expressed in reproductively mature females and has been widely used to assess the estrogenic potential of xenobiotics (Hutchinson, et al. 2006). Octylphenol has been reported to bind the estrogen receptors of a number of species (Gutendorf and Westendorf, 2001; Matthews, et al. 2000; Routledge and Sumpter ,1997) and induce vitellogenin in fish in vitro assays (Navas and Segner ,2006; White, et al. 1994). In this study, significant vitellogenin induction was observed during the larval sampling at the highest octylphenol treatment (Fig. 7) which is consistent with its mechanism of action. While a higher level of vitellogenin was measured in adult males at this concentration, the levels

were not statistically significant (Fig. 7). The discrepancy between the larval and adult responses could be due to 1) a difference in the physiology of the liver between tadpoles and adult frogs such that the adult liver is less inducible than the larval, 2) a difference in target tissue dosimetry due to differences in absorption, distribution, metabolism, and elimination between the two life stages such that the adult liver is seeing relatively less chemical, or 3) the larval response is less variable than the adult response and due to purely statistical differences or biological differences such as endogenous hormonal status. While sperm count was significantly decreased in adult frogs exposed to 11 µg/L OP there was no significant effect seen in the 36 µg/L OP exposed frogs. However, a significant increase in multifocal testicular germ cell sloughing was seen in 1 µg/L and 36 µg/LOP exposed frogs, indicating that OP exposure may be disrupting spermatogenesis, but is not sufficient to reduce sperm count. Studies in fish have shown the presence of testis-ova in medaka exposed to 11.4 µg/L OP (Seki, et al. 2003; Seki, et al. 2002), decreased gonadosomatic index and increased testis-ova in eelpout exposed to 10 µg/L OP (Rasmussen, et al. 2005b), and decreased late sperm development and increased testis anomalies in sheepshead minnows exposed to 33.6 µg/L OP (Karels, et al. 2003), indicating that *X. tropicalis* testes are less sensitive to the effects of OP exposure than fish.

In this study we observed the presence of male frogs with oviducts in all OP exposed groups, 1 each in the 1.0 (n=36) and 3.3 µg/L (n=18) OP exposed groups, 4 in the 11 µg/L OP exposed group (n=17), and 12 in the 36 µg/L OP exposed group (n=18), but not in the controls (n=93). The oviducts were smaller than those seen in females, but there was a significant increase in male oviduct weight in the 36 µg/L exposed frogs compared to the 11 µg/L OP exposed frogs, indicating a possible concentration – dependent relationship between OP concentration and male oviduct weight. There were no significant differences in other endpoints between males with oviducts and normal males. In studies with *Xenopus laevis,* tadpoles exposed to polychlorinated biphenyls develop male oviducts and decreased larynx weight (Qin, et al. 2007), and tadpoles exposed to low concentration di-*n*-butyl-phthalate develop male oviducts

and testis histological abnormalities (Lee and Veeramachaneni, 2005), indicating that other weakly estrogenic EDCs can induce abnormal secondary sex characteristics in *Xenopus* species.

4.1. Conclusion

The primary focus of this study was to determine the effects of long term OP exposure on *Xenopus tropicalis* from NF stage 46 through sexual maturity. OP exposure induced elevated vitellogenin levels in pre-metamorphic froglets of both sex phenotypes, but had no effect on adult frog vitellogenin levels, indicating that premetamorphic froglets are more sensitive to the temporary effects of OP exposure than adults. We found that exposure to OP at environmentally relevant concentrations induced the presence of males with oviducts with both testes and oviducts in all exposed groups. OP exposure also had no effect on serum estradiol or testosterone levels, or on ovarian endpoints. Since our lowest observed effect concentration (LOEC) for development of male oviducts was 1 µg/L and environmental levels of OP have been measured as high as 348 ng/L in the Mai Po Marshes Nature Reserve in Hong Kong (Li, et al. 2007), the possibility of adverse reproductive effects on amphibians in the field may exist at OP contaminated sites. This study was a successful evaluation of the effects of a long term low concentration EDC exposure on *X. tropicalis*. Additionally this study shows that *X. tropicalis* can be used as an amphibian species for use in tier II amphibian estrogenic EDC exposure studies and that the experimental design using a flow-through diluter system and sampling animals at the penultimate stage of metamorphosis and after sexual maturity is capable of detecting significant effects from weak ER-α agonist exposure.

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Table 1. Water quality values.

Parameter	Set Point	Mean	Minimum	Maximum	N
Temperature (°C)	25	25.0	23.4	25.7	651
pH	7.5	7.5	7.25	7.77	651
Dissolved Oxygen (mg/L)	3.4 – 8.5	4.7	0.7[a]	7.1	651
Alkalinity as $CaCO_3$ (mg/L)	>50[b]	122	100	143	62
Hardness as $CaCO_3$ (mg/L)	>175[b]	198	176	218	62
Conductivity (Siemens/cmor)	500 – 1000[b]	666	571	760	630
Ammonia Nitrogen (mg/L)	< 1.8	0.83	0.22	3.18[c]	56

*N is the number of measurements taken over the course of the study.
[a]Transient reading on week 10, aeration was increased.
[b](Green, 2010)
[c]Tanks drained and refilled with fresh aquaculture water.

Table 2. Interim and Final Necropsy Somatic Data. Body length is defined as the distance from the tip of the snout to the cloacal vent. Liver, kidney, and gonad somatic indices were calculated as organ percent of total body mass. Data are represented as mean ± 1 S.D. of tank averages. For controls, n = 9, for OP groups, n = 3.

	Nominal Octylphenol Concentration (µg/L)				
	0	1.0	3.3	11	36
Larval Sub-Sample					
Male Body Mass (mg)	0.85 ± 0.13	0.86 ± 0.02	0.87 ± 0.01	0.88 ± 0.08	0.85 ± 0.07
Male Body Length (mm)	17.7 ± 0.6	18.2 ± 0.2	18.1 ± 0.3	18.0 ± 0.6	18.0 ± 0.3
Female Body Mass (mg)	0.81 ± 0.10	0.91 ± 0.16	0.89 ± 0.12	0.84 ± 0.02	0.88 ± 0.08
Female Body Length (mm)	17.5 ± 0.6	18.1 ± 0.8	17.8 ± 0.2	17.8 ± 0.2	18.3 ± 0.2
Final Sample					
Male Body Mass (g)	12.2 ± 0.7	12.4 ± 0.3	12.5 ± 0.9	12.4 ± 0.7	12.9 ± 0.7
Male Body Length (mm)	47.0 ± 1.4	48.0 ± 0.7	48.2 ± 0.8	48.2 ± 1.4	48.3 ± 1.4
Female Body Mass (g)	17.3 ± 0.6	16.9 ± 1.5	17.1 ± 0.2	16.3 ± 1.5	17.3 ± 1.4
Female Body Length (mm)	52.9 ± 0.6	52.3 ± 1.7	52.3 ± 0.5	52.2 ± 1.2	53.1 ± 1.3
Male Liver Index	2.8 ± 0.1	2.9 ± 0.1	3.1 ± 0.1	2.9 ± 0.3	2.8 ± 0.2
Male Kidney Index	0.49 ± 0.02	0.52 ± 0.02	0.49 ± 0.03	0.53 ± 0.04	0.41 ± 0.03
Testis Somatic Index	0.20 ± 0.01	0.22 ± 0.01	0.22 ± 0.02	0.20 ± 0.02	0.22 ± 0.01
Female Liver Index	2.9 ± 0.1	2.9 ± 0.2	2.8 ± 0.1	2.8 ± 0.3	2.8 ± 0.1
Female Kidney Index	0.56 ± 0.14	0.51 ± 0.03	0.47 ± 0.05	0.55 ± 0.04	0.46 ± 0.07
Ovary Somatic Index	6.3 ± 0.9	5.7 ± 0.7	5.4 ± 0.2	5.5 ± 0.7	5.0 ± 0.8

Table 3. Testis Histology Findings.

Concentration Groups					
	Control	**1**	**3.3**	**11**	**36**
n	13	13	6	7	13
Seminiferous Tubules					
Normal	7/13	0/13	2/6	2/7	0/13
Germ Cell Sloughing					
Focal					
Very mild	3/13	1/13	2/6		2/13
Mild	1/13				
Moderate					
Severe					
Multifocal		*			*
Very mild	1/13	1/13	1/6	2/7	2/13
Mild	1/13	5/13		2/7	2/13
Moderate		1/13			3/13
Severe		5/13	1/6	1/7	4/13

Very Mild = few observations; Mild = effect observed in 1/3 of the tissue; Moderate = effect observed in 2/3 of the tissue; Severe = effects observed in > 2/3 of the tissue. Descriptives were coded as 0 = normal, 1 = very mild, 2 = mild, 3 = moderate, and 4 = severe for statistical analysis.
* Indicates significant difference from control ($p \leq 0.05$).

Table 4. Ovary weights, oocyte numbers and oocyte staging at final necropsy. Data are represented as mean ± 1 S.D. of tank averages. For controls, n = 9, for OP groups, n = 3.

	Nominal Octylphenol Concentration (µg/L)				
	0	1.0	3.3	11	36
Ovary Weight (g)	1.10 ± 0.18	0.98 ± 0.17	0.93 ± 0.04	0.92 ± 0.14	0.89 ± 0.15
Oocyte Number X 1000	18.2 ± 4.4	15.4 ± 2.1	17.4 ± 1.6	16.6 ± 6.1	18.0 ± 2.5
Oocyte Staging (%)					
Stage I/II	64.7 ± 6.5	68.6 ± 7.3	64.9 ± 2.4	68.8 ± 6.9	70.8 ± 3.7
Stage III	5.0 ± 1.0	4.2 ± 0.5	4.8 ± 0.4	4.8 ± 1.1	5.3 ± 1.5
Stage IV	6.9 ± 2.3	5.7 ± 0.3	6.0 ± 0.3	6.6 ± 1.4	4.5 ± 1.0
Stage V/VI	22.3 ± 5.9	20.5 ± 6.1	23.2 ± 2.2	19.0 ± 8.1	18.4 ± 3.4
Atretic	1.1 ± 0.7	1.0 ± 0.7	1.1 ± 0.3	0.9 ± 0.6	1.0 ± 0.4

Table 5. Serum steroid hormone levels at final necropsy. Data are represented as mean ± 1 S.D. of tank averages. For controls, n = 9, for OP groups, n = 3.

	Nominal Octylphenol Concentration (µg/L)				
	0	1.0	3.3	11	36
Estradiol (ng/ml)					
Male	0.53 ± 0.05	0.53 ± 0.04	0.65 ± 0.17	0.55 ± 0.02	0.40 ± 0.05
Males with Oviducts		0.39[a]	BDL[ab]	0.75 ± 0.02	0.43 ± 0.03
Female	3.4 ± 0.15	3.4 ± 0.28	3.4 ± 0.11	3.7 ± 0.46	3.6 ± 0.08
Testosterone (ng/ml)					
Male	16.4 ± 1.1	14.6 ± 1.8	16.4 ± 4.0	19.3 ± 2.2	17.2 ± 3.6
Males with Oviducts		43.1[a]	8.8[a]	15.7 ± 0.8	20.1 ± 3.2
Female	5.4 ± 0.4	5.0 ± 0.7	4.3 ± 0.1	4.8 ± 0.9	4.6 ± 0.6

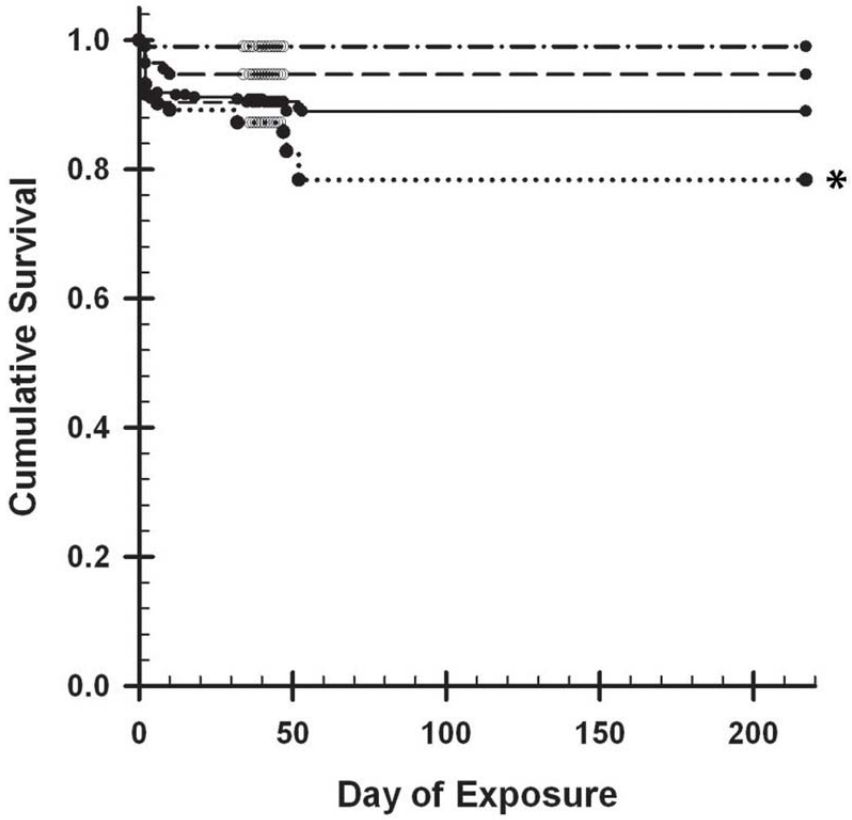
[a]Only one male with oviducts present in exposure group.
[b]Below detection limits.

Supplementary Table 1. Measured Octylphenol Tank Concentrations by Week of Exposure

	Nominal Octylphenol Concentration (µg/L)				
Week	**1.0**	**3.3**	**11**	**36**	**Action**
0	1.0 ± 0.1	4.5 ± 0.1	14.9 ± 1.0	44.3 ± 1.0	
1	0.8 ± 0.1	3.6 ± 0.1	11.9 ± 0.6	39.7 ± 2.3	
2	0.6 ± 0.3	2.5 ± 0.2	10.6 ± 0.1	32.2 ± 2.9	Disinfected diluter
3	1.6 ± 0.1	3.9 ± 0.2	10.2 ± 0.8	28.7 ± 2.3	
4	1.2 ± 0.3	2.7 ± 0.1	7.2 ± 0.7	28.3 ± 4.3	Disinfected diluter
5	0.5 ± 0.2	2.0 ± 0.3	6.1 ± 0.8	23.9 ± 3.5	
6	2.8 ± 0.3	5.1 ± 0.5	12.4 ± 1.9	42.5 ± 2.1	Disinfected diluter, replaced tanks
7	1.1 ± 0.1	4.2 ± 0.3	14.6 ± 0.2	51.0 ± 0.4	
8	1.0 ± 0.1	3.6 ± 0.6	10.6 ± 0.5	38.1 ± 5.2	Disinfected diluter
9	2.1 ± 0.2	5.2 ± 0.2	11.6 ± 1.4	31.2 ± 4.1	
10	0.7 ± 0.1	2.3 ± 0.4	6.6 ± 1.8	19.7 ± 1.8	Disinfected diluter
11	1.7 ± 0.1	3.7 ± 0.5	7.2 ± 4.6	35.6 ± 2.5	
12	0.9 ± 0.2	3.0 ± 0.5	8.7 ± 2.9	37.8 ± 3.5	Disinfected diluter, replaced tank 5*
13	1.6 ± 0.1	4.1 ± 0.4	9.9 ± 1.8	40.9 ± 6.3	Replaced all tanks
14	1.3 ± 0.0	2.7 ± 0.1	7.6 ± 0.6	25.8 ± 1.4	Disinfected diluter
15	0.7 ± 0.1	3.2 ± 0.3	9.7 ± 0.1	33.5 ± 3.1	
16	0.5 ± 0.1	3.4 ± 0.2	11.2 ± 0.2	41.6 ± 3.4	Disinfected diluter
17	1.3 ± 0.2	3.1 ± 0.3	11.5 ± 0.3	41.6 ± 0.7	
18	1.5 ± 0.2	3.6 ± 0.1	11.8 ± 0.8	36.1 ±11.6	Disinfected diluter
19	1.2 ± 0.1	3.3 ± 0.2	11.1 ± 0.4	39.9 ± 4.0	
20	1.1 ± 0.2	2.8 ± 0.2	10.5 ± 0.7	35.5 ± 2.9	Disinfected diluter
21	1.3 ± 0.1	3.5 ± 0.3	9.6 ± 0.6	33.1 ± 0.6	
22	1.1 ± 0.1	2.8 ± 0.2	6.7 ± 0.2	27.2 ± 2.0	Disinfected diluter
23	0.9 ± 0.2	3.3 ± 0.3	12.6 ± 1.1	34.3 ± 1.9	
24	2.0 ± 0.9	3.1 ± 0.1	9.8 ± 0.8	31.1 ± 0.9	Disinfected diluter
25	1.3 ± 0.4	3.7 ± 0.1	12.3 ± 0.6	37.5 ± 3.6	
26	0.6 ± 0.2	3.8 ± 0.1	12.2 ± 0.5	40.1 ± 2.3	Disinfected diluter
27	1.8 ± 0.1	3.5 ± 0.1	13.2 ± 0.4	42.5 ± 4.4	
28	1.4 ± 0.2	3.5 ± 0.1	10.0 ± 1.6	39.9 ± 5.4	Disinfected diluter
29	1.2 ± 0.1	3.6 ± 0.2	11.4 ± 0.4	38.5 ± 3.5	
30	1.6 ± 0.5	3.1 ± 0.1	10.0 ± 0.3	32.4 ± 2.1	Disinfected diluter
31	1.7 ± 0.2	4.8 ± 0.3	11.9 ± 0.6	47.5 ± 1.8	
Mean	1.3 ± 0.5	3.5 ± 0.7	10.5 ± 2.2	35.9 ± 7.1	
Range	0.5 – 2.8	2.0 – 5.2	6.1 – 14.9	19.7 – 51.0	

*Tank 5 was an 11 µg/L OP exposure tank that contained significantly less OP (5.4 µg/L) than the other 11 µg/L OP exposure tanks, and more organic debris than all the other tanks at week 12.

Figure 1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Cumulative Survival
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200
Day of Exposure
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Figure 2

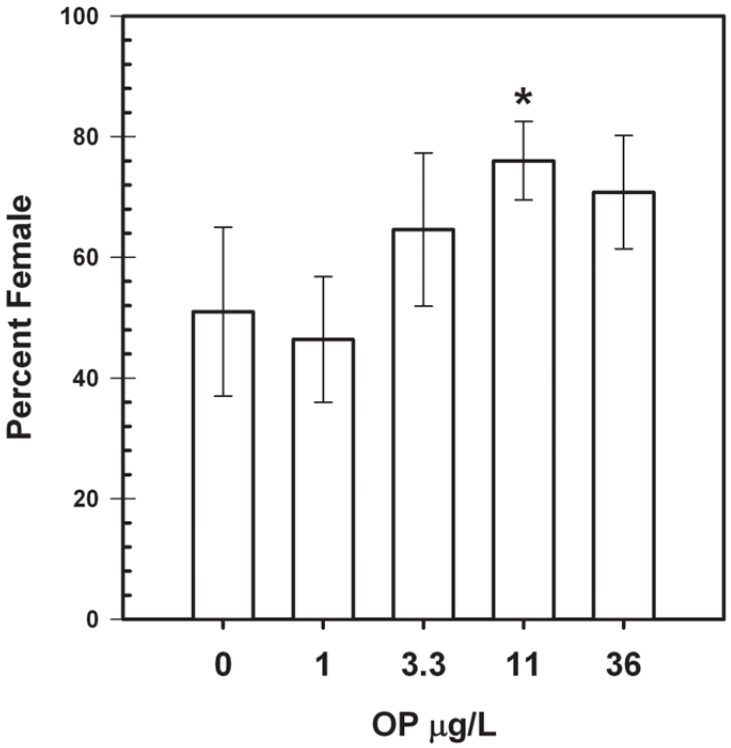

Figure 3

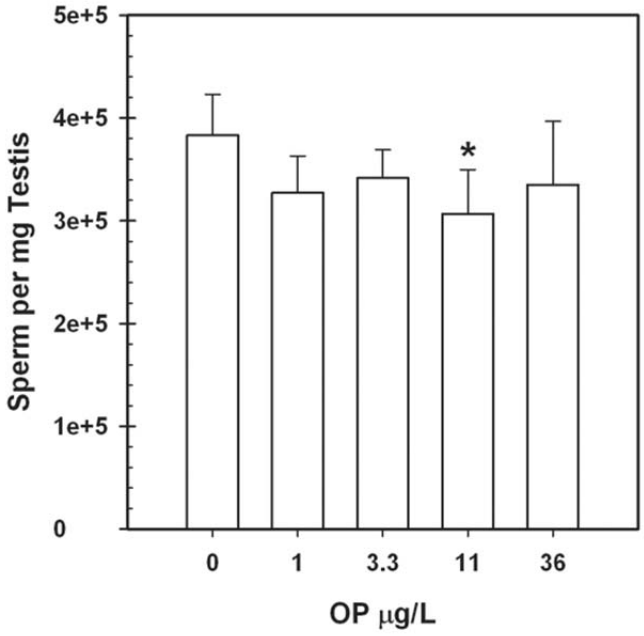

Figure 4

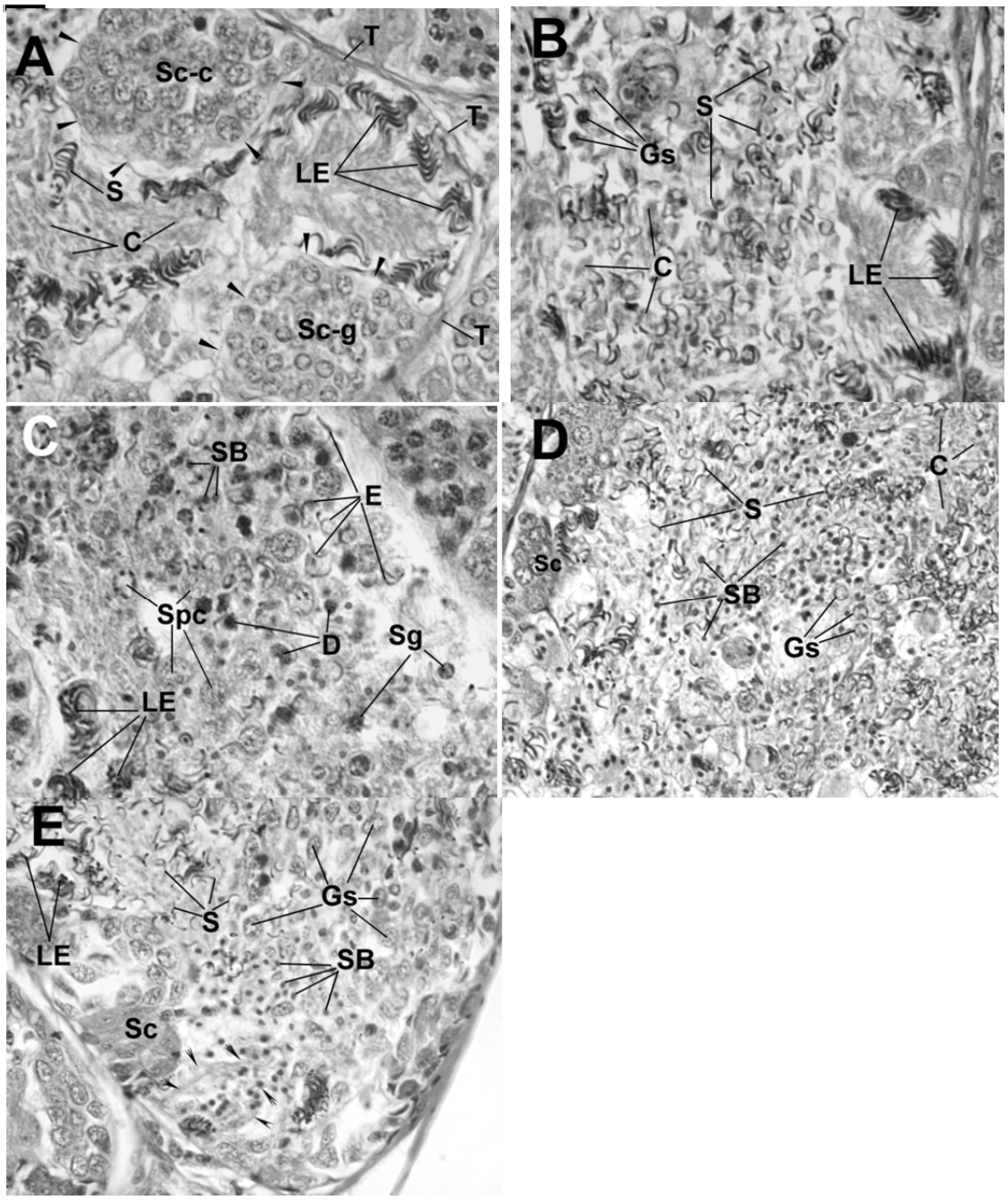
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Figure 5

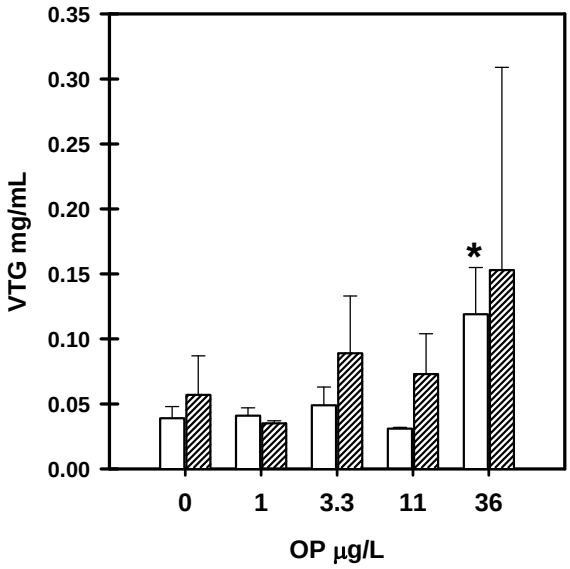

Figure 6

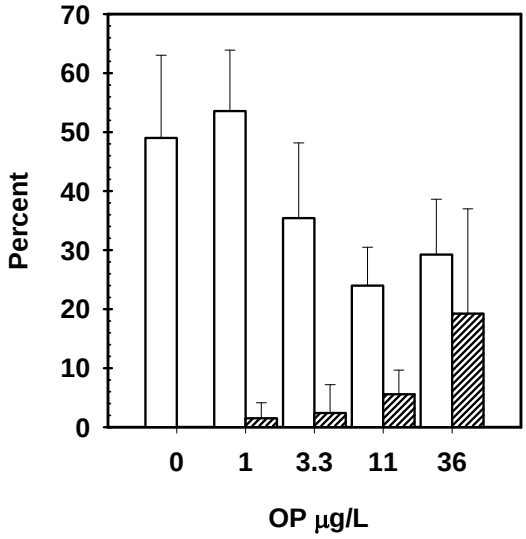

Figure 7

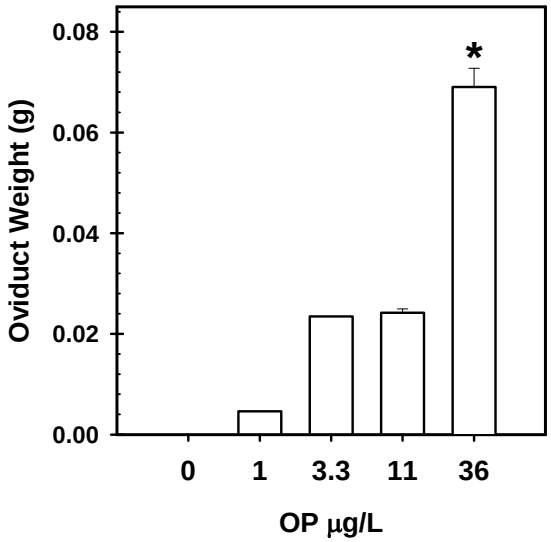

Figure Legends

Figure 1. Kaplan-Meier log rank plot of cumulative survival. Animals euthanized for interim and final necropsy, and accidental deaths were treated as censored data. Control (solid), 1.0 µg/L OP (long dash), 3.3 µg/L OP (dots), 11 µg/L OP (dot dash), and 36 µg/L OP (short dash) groups were plotted as probability of survival for each day of exposure. * indicates a statistically significant difference between OP exposed groups and control ($p \leq 0.001$) by pairwise multiple comparison procedure (Holm-Sidak method).

Figure 2. Phenotypic sex distribution at week 31 of exposure by OP concentration. * indicates a statistically significant difference between OP exposed groups and control ($p \leq 0.05$).

Figure 3. Million sperm per mg testis at week 31 of exposure. * indicates a statistically significant difference between OP exposed groups and control ($p \leq 0.05$).

Figure 4. Photomicrograph showing a portion of a control seminiferous tubule. C = residual spermatid cytoplasm; LE = late elongate spermatid bundles; S= sperm; Sc-c = spermatocysts containing spermatocytes; Sc-g = spermatocysts containing spermatogonia = boundary layer of the seminiferous tubule; Arrow heads = outer boundary of spermatocysts (4A).
Photomicrograph showing released sperm within the seminiferous tubule lumen of control animals. C = residual spermatid cytoplasm; Gs = sloughed degenerating germ cells; LE = late elongate spermatid bundles; S= sperm (4B).
Photomicrograph of the contents of the seminiferous tubule lumen of high concentration animals. Note the sloughed germ cells (Gs), elongated spermatids (E) retaining their residual cytoplasm and sperm (S) (Figures 4C and 4D). Spherical bodies (SB) were observed within the tubular lumen of many seminiferous tubules (4D).

Photomicrograph of the contents of the seminiferous tubule lumen of high concentration animals. Note the sloughed germ cells (Gs), bundles of late elongated spermatids entrenched with their Sertoli cells (LE) and sperm (S). This photomicrograph suggests that the spherical bodies are underdeveloped germ cells, possibly rounded spermatids, prematurely released from their spermatocysts. Arrow heads = outer boundary of the spermatocyst; Sc = spermatocyst containing early spermatogonia (4E).

Figure 5. Vitellogenin (VTG) levels for both sexes at stage 65 (☐) and male VTG levels at week 31 of exposure (▨).* indicates a statistically significant difference between OP exposed groups and control ($p \leq 0.05$).

Figure 6. Percent males with oviducts at week 31 of exposure.

Figure 7. Oviduct weights in males with oviducts at week 31 of exposure. * indicates a statistically significant difference between the 11 µg/L OP exposed group and the 36 µg/L OP exposed group ($p \leq 0.05$)